Multiple Conductance Channel Activity of Wild-Type and Voltage-Dependent Anion-Selective Channel (VDAC)-Less Yeast Mitochondria

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ABSTRACT Yeast mitoplasts (mitochondria with the outer membrane stripped away) exhibit multiple conductance channel activity (MCC) in patch-clamp experiments that is very similar to the activity previously described in mammalian mitoplasts. The possible involvement of the voltage-dependent anion-selective channel (VDAC) of the outer membrane in MCC activity was explored by comparing the channel activity in wild-type yeast mitoplasts with that of a VDAC-deletion mutant. The channel activity recorded from the mutant is essentially the same as that of the wild-type in the voltage range of −40 to 30 mV. These observations indicate that VDAC is not required for MCC activity. Interestingly, the channel activity of the VDAC-less yeast mitoplasts exhibits altered gating properties at transmembrane potentials above and below this range. We conclude that the deletion of VDAC somehow results in a modification of MCC's voltage dependence. In fact, the voltage profile recorded from the VDAC-less mutant resembles that of VDAC.

INTRODUCTION

Channels exist in both the inner and outer mitochondrial membranes, where they could provide a pathway for the movement of materials (including protein) between the matrix and the cytoplasm and could be involved in the regulation of cellular metabolism. All of the inner membrane channels studied so far are normally quiescent (Kinnally et al., 1992) and, hence, should not interfere with oxidative phosphorylation under physiological conditions. Although the outer membrane channel voltage-dependent anion-selective channel (VDAC, also referred to as mitochondrial porin) has been well characterized on both a molecular and functional level (Mannella et al., 1992), those of the inner membrane are not as well understood.

Patch-clamp studies of mitoplasts (mitochondria treated to expose the inner membrane) have allowed the functional characterization of a variety of channels in their native membranes (Kinnally et al., 1989, 1992; Sorgato et al., 1987; Petronilli et al., 1989; Antonenko et al., 1994). The highest conductance channel class recorded from mitoplasts is the multiple conductance channel activity (MCC) (Kinnally et al., 1989) (also referred to as mitochondrial mega-channel (MMC) (Petronilli et al., 1989)), which has a peak conductance of 1000–1500 pS. In mammals, MCC activity is thought to correspond to the calcium-induced permeability transition pore (Szabó et al., 1992), which may play a role in ischemia-reperfusion injury (Gunter and Pfeiffer, 1990). The protein(s) responsible for MCC activity are not known, although a gap junction-like complex with pores in series at contact sites has been postulated, which would provide a pathway for solutes across both the inner and outer membranes (Kinnally et al., 1992). Pharmacological evidence suggests the involvement of the mitochondrial benzodiazepine receptor (mBzR) in mammalian MCC activity (Kinnally et al., 1993). The mBzR constituents are VDAC and a 18-kDa protein in the outer membrane, which are thought to complex with the adenine nucleotide translocator of the inner membrane (McEnery et al., 1992). Also, Szabó et al. (1993a, b) have speculated that a dimer of VDAC may be responsible for MCC activity.

This study confirms the presence of MCC activity in yeast. The conservation of this channel activity in two widely separated organisms, mammals and yeast, suggests a vital, yet unknown, function for MCC activity. Although we did record a 45 pS, slightly anion-selective activity that may be related to alkaline pH-activated anion activity (AAA) (Antonenko et al., 1994), we found no evidence in yeast of mitochondrial centum picoSiemen channel (mCS) activity, another mammalian mitoplast channel activity (Sorgato et al., 1987; Kinnally et al., 1993).

Comparisons of the mitoplast channel activity from wild-type yeast and a VDAC-deletion mutant are used to evaluate the possible involvement of VDAC in MCC activity in this study. The channel activity recorded from the mutant yeast mitoplasts is almost indistinguishable from the wild-type activity between −40 and 30 mV, but is different at higher potentials. In fact, the voltage profile recorded from the VDAC-less mutant resembles that of VDAC in that occupation of the fully open state is reduced at high potentials of either polarity (e.g., ±60 mV). Kinetic models are presented

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Abbreviations used: MCC, multiple conductance channel activity; VDAC, voltage-dependent anion-selective channel; mBzR, mitochondrial benzodiazepine receptor; PMSF, phenylmethylsulfonylfluoride; PSC, peptide-sensitive channel; AAA, alkaline pH-activated anion channel activity; mCS, mitochondrial centum picoSiemen channel.

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to summarize the single-channel data. A preliminary report of these findings has been made (Lohret and Kinnally, 1994).

MATERIALS AND METHODS

Yeast strains and mitochondrial preparation

Two strains of *Saccharomyces cerevisiae* were generously provided by M. Forte (Oregon Health Science University, Portland, OR). Strain M-3 is wild-type and strain M22–2 is a mutant in which the VDAC gene has been deleted and replaced with the leu2 gene (Blachly-Dyson et al., 1990). Cells are grown in 1% yeast extract, 2% peptone, and 2% raffinose at 30°C for 15–20 h with a yield of ≈4–6 g/l.

Mitochondria are isolated by a modification of the method of Daum et al. (1982). Yeast cells are sedimented at 3000 × *g* (5000 rpm in an SS-34 rotor) for 5 min and washed with water. The cells (0.5 g wet weight/ml 0.1 M $TrisSO_4$, pH 9.4, 10 mM dithiothreitol) are shaken at 30°C for 15 min. Cells are sedimented at 3000 × *g* for 5 min, washed in 1.2 M sorbitol, and resuspended in 1.2 M sorbitol, 20 mM KH_2PO_4, pH 7.4, to 0.15 g cells/ml. Cell walls are treated by incubation at 30°C with Zymolase 5000 (2 mg/g cells) for 30–40 min. Occasionally, cell walls are broken by vortexing 12–14 times for 40 s in the presence of an equivalent weight of 0.5-mm glass beads. Conversion to spheroplasts is monitored visually by lysis in distilled water. Spheroplasts are sedimented at 3000 × *g* for 5 min, washed in 1.2 M sorbitol, and resuspended to 0.15 g of cells/ml in homogenization buffer (HB, 0.6 M Sorbitol, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.2% BSA, 1 mM PMSF) for 10 min. The cells are homogenized by 10–15 strokes, diluted with 1 vol of HB, and debris is removed by a spin at 3000 × *g* for 5 min. Mitochondria are sedimented at 12,000 × *g* for 10 min, washed in HB and, finally, resuspended in 1× medium (70 mM sucrose, 230 mM mannitol, 5 mM HEPES, pH 7.4).

Mitoplasts are prepared by brief exposure to 5 mM HEPES on the slide followed by perfusion with 4 ml of patching medium (150 mM KCl, 5 mM HEPES, 1 mM EGTA, 1.05 mM $CaCl_2$, pH 7.4). Alternatively, and with the same results, mitoplasts are made by the French press method (Decker and Greenawalt, 1977) or by osmotic treatment with 5 mM HEPES as described previously (Kinnally et al., 1993; Campo et al., 1992). The mitoplasts are sedimented at 12,000 × *g* for 10 min and resuspended in 1× medium. Mouse heart and liver mitoplasts are isolated as reported previously (Kinnally et al., 1989, 1993).

Patch-clamping

High resistance (GΩ) seals form either spontaneously upon touching the mitoplast with the pipette or after applying a slight negative pressure. Patches are excised by lifting the pipette away from the mitoplast attached to the slide after a gigaseal is formed. The bathing medium and the solution in the patching pipettes is 0.15 M KCl, 5 mM HEPES, 1 mM EGTA, 1.05 mM $CaCl_2$, pH 7.4, at room temperature (~25°C). The reference electrode consists of a Ag-AgCl wire connected to the bath through a bridge containing the medium and 2% agar. Patch pipettes are pulled with a three-step program (courtesy A. K. Dean, Sutter Instruments, Novato, CA) on a Sutter model PC-84 puller to a resistance of 20–40 MΩ and a tip diameter of ~0.4 μM.

The conditions and procedures used are essentially the same as reported previously (Kinnally et al., 1993; Campo et al., 1992). Voltage clamp conditions are maintained with a Dagan 3900 or 8900 (Minneapolis, MN) patch-clamp amplifier. The voltage is reported relative to the mitochondrial matrix in excised patches, where $V = V_{bath} - V_{pipette}$ in the inside out mode. Unless otherwise stated, current is reported relative to zero current level measured at 0 mV under symmetrical ionic conditions and, therefore, is not leak-subtracted. Voltage and current traces are digitized with a NeuroData Neurocorder model DR390 (New York, NY) and stored on videotape. Computer analysis of current signals is bandwidth-limited to 2 kHz with a low pass filter (model 902 Frequency Devices, Haverhill, MA) and sampled at 5 kHz unless otherwise stated. Analysis usually is done with Strathclyde Electrophysiological Software (PAT, courtesy of J. Dempster, University of Strathclyde, Strathclyde, U.K.) through a DT2801A (Data Translation, Marlboro, MA) analog-digital board. Open probability is calculated as the percent of the total time spent in the fully open state or, when stated, a specific conductance level from amplitude histograms of current traces, usually of 20- to 40-s duration. Although not examined routinely, the open probability generally is unchanged when measured with longer durations, e.g., 100 s. Gating charge and V_0 are calculated from the voltage profiles of the open probability using the method of Moczydlowski (1986) as in Fig. 5. V_0 is the *x*-intercept, whereas gating charge is calculated from the slope of $\ln(P_0/(1 - P_0)$ plots as a function of voltage.

Reconstitution

Outer membranes are purified using the method of Mannella (1982), and the pellet from the gradient spin is used as the inner membrane preparation. Both preparations are reconstituted into giant liposomes (Sigma Type II-S soybean L-α-phosphatidylcholine) using the dehydration-rehydration method of Criado and Keller (1987) in 0.15 M KCl, 5 mM HEPES, pH 7.4, as described previously (Campo et al., 1992; Costa et al., 1991). In some mutant reconstitutions, the inner membrane fraction was solubilized in 0.05% lauryl maltoside detergent on ice for 1 h and reconstituted according to the method of McEnery et al. (1984).

SDS gel electrophoresis and Western blot

Gel electrophoresis of whole wild-type and mutant mitochondria is carried out on 12% acrylamide gels according to Laemmli (1970). Total protein is visualized with Bio-Rad silver stain kits (Bio-Rad Laboratories, Melville, NY). Western blot analysis is performed on both wild-type and mutant mitochondria. Proteins are transferred to nitrocellulose and probed with antibodies to amino acids 255–272 of *Neurospora crassa* VDAC (gift of C. Mannella, Wadsworth Center, Albany NY) using the method of Towbin et al. (1979) and visualized with an AuroProbe BLplus secondary antibody reaction (Amersham, Amersham, U.K.). The *Neurospora crassa* antibody is known to cross-react strongly with yeast VDAC (Stanley, 1994).

RESULTS

VDAC has been linked to inner mitochondrial membrane channel activity by pharmacological studies and other data (see Kinnally et al., 1993; Kinnally and Tedeschi, 1994). In addition, Szabó et al. (1993a, b) have proposed a major role for VDAC in MCC activity. A direct comparison of the behavior of wild-type MCC activity with that recorded from mutant yeast (strain M22–2) mitoplasts from which the VDAC gene has been deleted enables a closer evaluation of the possible role of VDAC in MCC activity. Western blot analysis (Fig. 1) confirms the absence of VDAC in the mutant yeast strain M22–2 mitochondria used for these experiments. The *Neurospora crassa* VDAC antibody reacts with a protein of the wild-type yeast mitochondria at 31,000 kDa (Stanley, 1994); as expected, no such band is seen with the

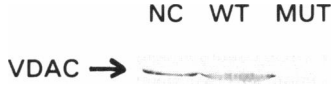

FIGURE 1 VDAC is absent in M22–2 yeast mitochondria. Western blot analysis using an antibody to VDAC (amino acids 255–272) of *Neurospora crassa* (NC) shows the cross-reactivity with VDAC in the wild-type (WT) yeast and the absence of VDAC in strain M22–2 VDAC-less mutant (MUT) yeast mitochondria.

VDAC-less mutant strain M22–2 mitochondria. Silver-stained mitochondrial protein gels of mutant and wild-type yeast reveal very complex patterns of bands (data not shown), and no meaningful differences are resolved.

MCC activity was described originally for mammalian mitoplasts (mitochondria with the inner membrane exposed) using patch-clamp techniques. It is defined operationally as a single class of many conductance levels (some with a slight cation selectivity) and a peak conductance of 1000–1500 pS (Kinnally et al., 1989, 1992). It is recorded from 76% of the patches (n = 50 mouse liver mitoplast patches, Kinnally et al., 1991), but the incidence varies with method of mitochondrial isolation. Although the transition sizes vary from 50 to 1500 pS, those of 300–500 pS are most prevalent. NanoSiemen transitions are recorded from 20% of the patches (n = 25 rat heart mitoplasts, Zorov et al., 1992).

The principal channel activity recorded from yeast mitoplasts is very similar to that recorded from its mammalian counterpart. The activity recorded from 76% of the wild-type yeast (strain M-3) mitoplast patches (n = 21 patches) and 58% of the mutant yeast (strain M22–2) mitoplasts (n = 48 patches) has a peak conductance of 1000–1500 pS as well as multiple conductance levels. The peak conductance (1300 pS in Figs. 2 and 3) and some of the subconductance levels (e.g.,

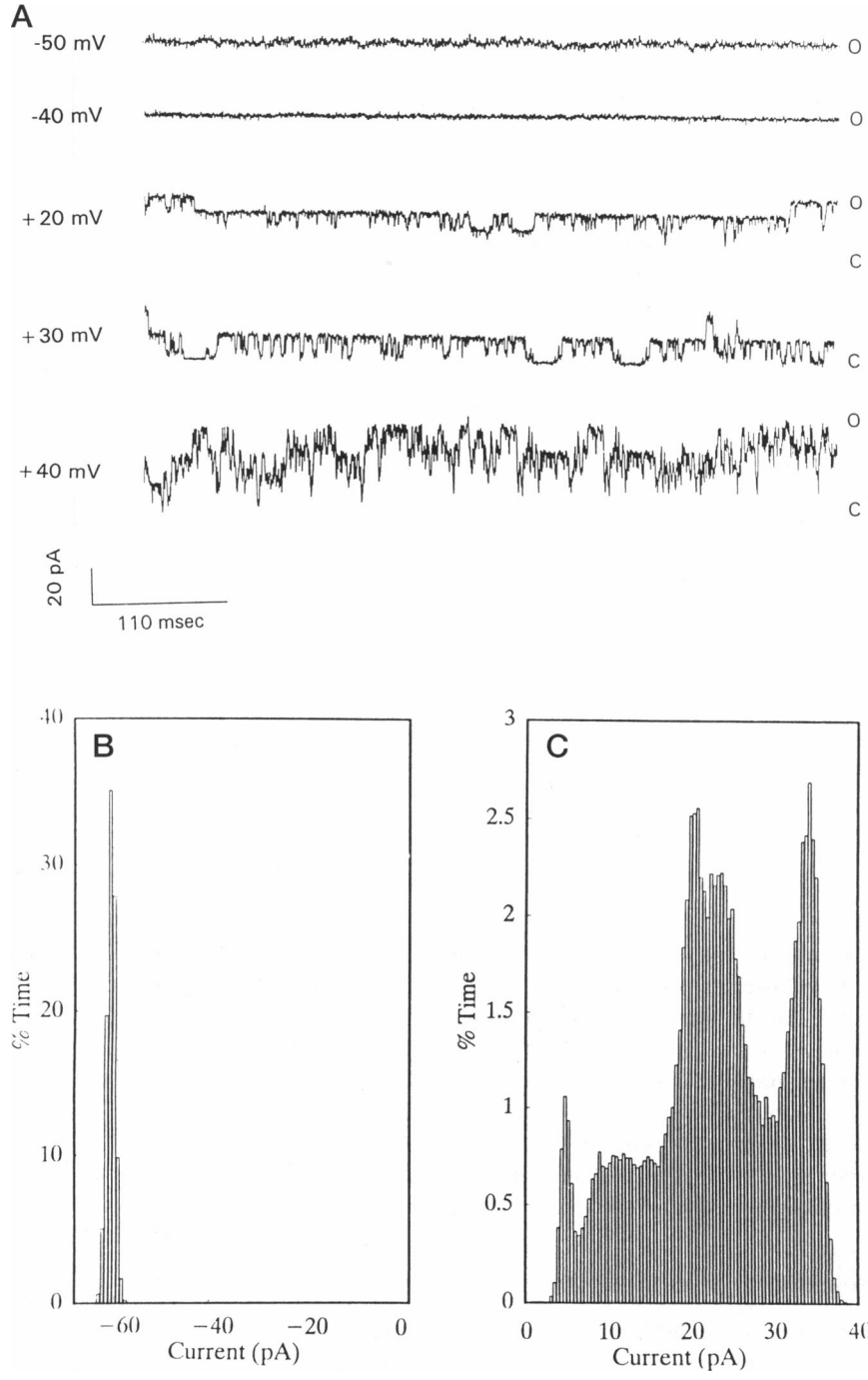

FIGURE 2 MCC activity is recorded from wild-type yeast mitoplasts. (*A*) Current traces at selected voltages were recorded from an excised mitoplast patch. Current data is bandwidth-limited to 2 KHz (sampled at 5 kHz). Patching medium is symmetrical 0.15 M KCl, 5 mM HEPES, 1 mM EGTA, 1.05 mM $CaCl_2$ ($\sim 10^{-5}$ M free Ca^{2+}), pH 7.4. The closed (C) and open (O) states are as indicated. (*B*) Amplitude histogram shows the occupation of the fully open state (1300 pS) at −40 mV is almost constant as indicated as the % of the total time per bin. (*C*) Amplitude histogram shows the occupation of multiple conductance levels at 30 mV. In general, amplitude histograms are generated from current traces of 10–60 s in duration and have 0.4 pA binwidths. Current levels are not leak-corrected, i.e., current levels are reported relative to the zero current level (0 pA) measured at 0 mV under symmetrical ionic conditions.

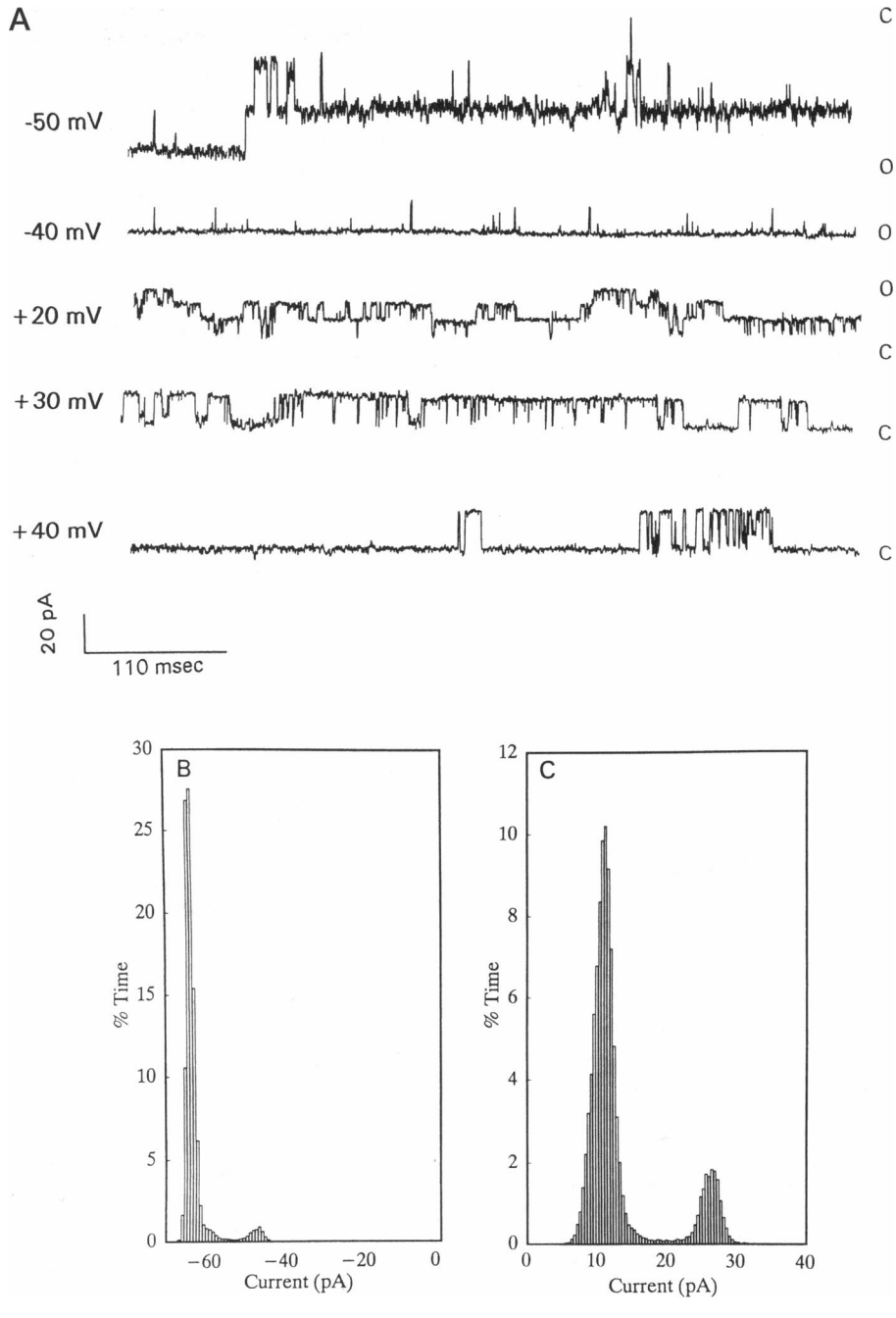

FIGURE 3 MCC activity is recorded from mitoplasts isolated from VDAC-less mutant yeast. (*A*) Current traces recorded from an excised VDAC-less mutant yeast mitoplast patch are shown for selected voltages. Amplitude histograms show occupation of (*B*) the fully open state (1300 pS) at −40 mV and (*C*) multiple conductance levels at 30 mV. Other conditions as in Fig. 2.

500 and 900 pS) are shown in the current traces and amplitude histograms of Fig. 2 *A-C* for the wild-type and Fig. 3 *A-C* for the mutant. The predominate transition sizes for both yeast strains are 300–500 pS. The enormous nano-Siemen transitions characteristic of mammalian MCC activity occurring between the fully open (1000–1500 pS) and closed states are present in 19% and 18% of the fully analyzed wild-type (n = 16) and mutant (n = 28) patches, respectively (see Fig. 4 for sample current traces).

Selectivity

Like mammalian MCC, which has a slight cation selectivity in some substates (Kinnally et al., 1989), yeast MCC is slightly cation-selective (n = 4 wild-type and n = 8 mutant patches). The yeast activity's reversal potential in the presence of a 150:30 mM KCl gradient is ~25 mV, which corresponds to relative permeability ratios for K^+/Cl^- of ~6 (n = 4 wild-type and n = 4 mutant patches, data not shown).

Voltage dependence

Voltage-dependent behavior is observed in about 95% of the mitoplast patches exhibiting multiple conductance channel activity (n = 16 wild-type patches and n = 28 mutant patches). There is no significant difference between the wild-type and mutant voltage profiles of the level occupancy probability in the range of −40 to 30 mV as shown in Figs. 5 and

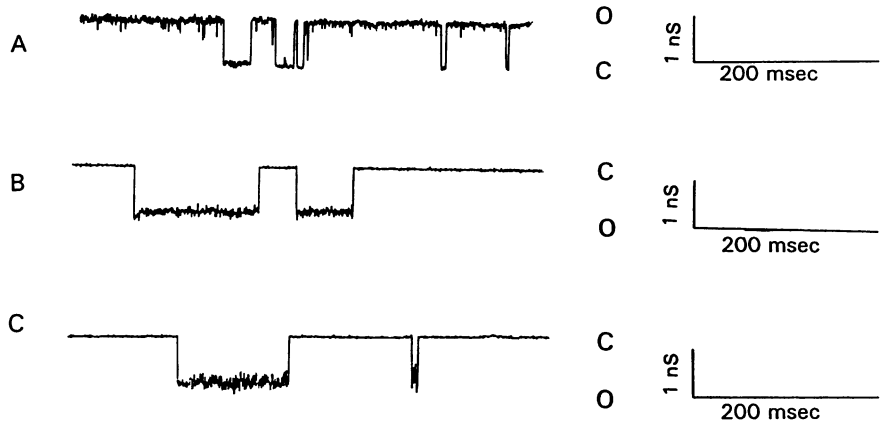

FIGURE 4 Current traces reveal nanoSiemen transitions in MCC activity from mouse and yeast mitoplasts. Current traces recorded from excised patches at (*A*) 20 mV from mammalian (rat heart), (*B*) −30 mV from wild-type, and (*C*) −40 mV from VDAC-less mutant yeast mitoplasts show high conductance (nS) transitions with a bandwidth of 4 kHz (10-kHz sampling). Mammalian patching medium is symmetrical 0.15 M KCl, 5 mM HEPES, 1 mM EGTA, 0.75 mM $CaCl_2$, pH 7.4. All other conditions as in Fig. 2.

6. MCC occupies the fully open state (~1300 pS) almost exclusively at −40 mV, as shown in the amplitude histograms of Figs. 2 *B* and 3 *B*. Transitions from the peak to lower conductance levels (e.g., 500 and 900 pS) and the closed state are observed most frequently and are longer in duration at low positive potentials, as shown in the current traces and amplitude histograms of Figs. 2 and 3 as well as the substate voltage profiles of Fig. 6.

Determination of the effective gating charge (measure of the functional charge that moves across the membrane to open or close the channel) and V_0 (voltage where the probability of occupying the open and closed states are equal, i.e., 0.5) allows quantification of the voltage dependence of the peak state of this multiple conductance channel activity. The open probability of the peak conductance level approaches 1 at low negative voltages and declines rapidly with the application of low positive potentials, as shown in Fig. 5 *A*. The V_0 and effective gating charge determinations calculated at low positive potentials for the peak conductance level (from the *x*-intercept and slope in Fig. 5 *C*) for the wild-type and mutant are not distinguishable (V_0 of 5.3 ± 8 and 6.3 ± 10 mV and gating charges of −4.7 ± 1.1 and −4.7 ± 1.3 for the wild-type and mutant activities, respectively (mean ± SD, n = 4 patches)). (Note that in one mutant patch, a V_0 of −35 mV was observed, suggesting a significant variability in this parameter, which has been noted in other systems (Hartshone et al., 1986).) A shifted V_0 (23 ± 3 mV) but very similar gating charge (5.4 ± 1.0 (mean ± SD, n = 4 patches)) are found with mammalian MCC activity (data not shown).

The voltage profile of the channel activity recorded from mutant yeast mitoplasts is almost indistinguishable from that of the wild-type between −40 and 30 mV. However, significant differences in conductance level occupancy are noted at higher potentials, as shown in Figs. 5 and 6. In particular, the mutant MCC open probability is significantly lower then the wild-type outside the −40 to 30 mV range. Although the probability of occupying the 900 pS or peak conductance levels is higher with larger positive potentials in the wild-type activity, that of the mutant remains low. At potentials more negative than −40 mV, the mutant activity open probability is reduced, whereas that of the wild-type remains high. Actually, the voltage profile recorded from the VDAC-less mutant resembles that of VDAC in that occupation of the fully open state is reduced at high potentials of either polarity (e.g., ±60 mV).

Single-channel analysis

Analysis of current traces containing single-channel events permits further comparisons of wild-type and mutant behavior. The mean open and closed times reflect the voltage dependence of the multiple conductance channel activity, as shown in Fig. 7. The decrease in open probability at low positive potentials (~10 to 30 mV) relative to high negative potentials is associated with both a decrease in mean open time and an increase in mean closed time. The wild-type mean open time is seconds long at −40 mV and is considerably shorter in the mutant, e.g., 39 ms. Although the fully open state is observed rarely in the mutant at 40 mV, the wild-type mean open time is 1.5 ms. The fully closed state usually is not observed at negative potentials. The mean closed time increases for both the wild-type and mutant at low positive potentials. Higher positive potentials result in a further increase in closed time for the mutant (e.g., 242 ms at 40 mV), whereas a decrease in closed time is noted for the wild-type (5 ms at 40 mV). Substate times were not determined because of the complexity of multiple subconductance levels.

Open and closed time distributions usually could be best-fit with two to three exponentials at different voltages, supporting the existence of multiple kinetic states (Campo et al., 1992; Moczydlowski, 1986), as shown in Fig. 8. This analysis is restricted because not all states are seen at all voltages, e.g., the closed state rarely is seen at larger negative potentials (e.g., −40 mV). Fast components (τ < 3 mS), representing flickering in current traces, as well as longer duration components (up to seconds) are present in the open and closed state time distributions of both mutant and wild-type preparations. The kinetic states include short duration (τ < 3 ms) open and closed states (O_S and C_S) and intermediate duration (τ = 3–100 ms) open and closed states (O_I and C_I), as shown in Fig. 8. A seconds-long duration open state (O_L) is seen particularly at high negative potentials in the wild-type and can be visualized by the current traces at −40 and

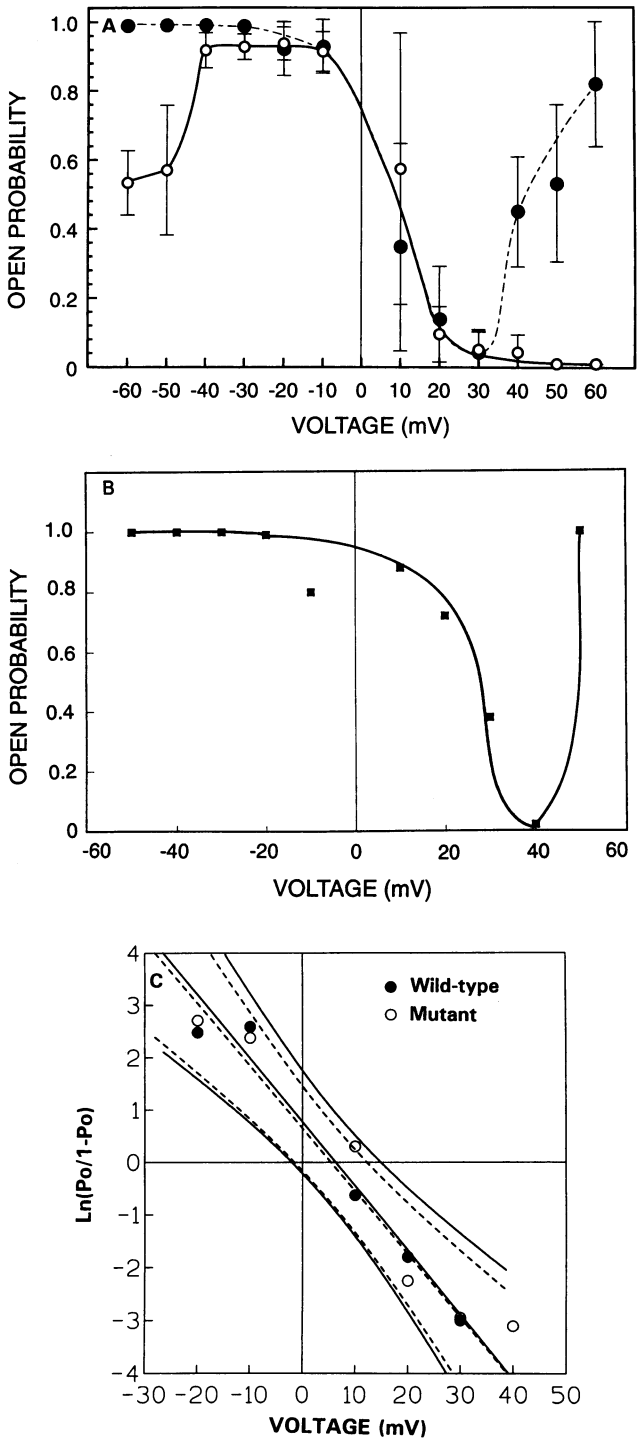

FIGURE 5 Open probability of the 1000–1400 pS conductance level of (*A*) yeast (wild-type yeast (●) and VDAC-less mutant yeast (○)) and (*B*) mouse mitoplast MCC activity is voltage-dependent. Total amplitude histograms are used to calculate open probability (P_0, as the ratio of the time spent at the peak conductance level relative to the total time at various voltages for MCC activity. Current records are taken for generally 20–40 s after holding the potential at zero mV for 5–10 s. Data shown for yeast MCC is mean ± SD for four different patches from each type of mitoplast. Other conditions as in Fig. 2. See Fig. 6 for substate occupation. (*C*) The open probability is transformed to a linear function over a range of voltages by plotting $\ln(P_o/1 - P_o)$. Linear regression lines plus 95% confidence limits are plotted for both wild-type (- - - -) and mutant (——). The slope is used to calculate gating charge, and the *x*-intercept indicates the V_0.

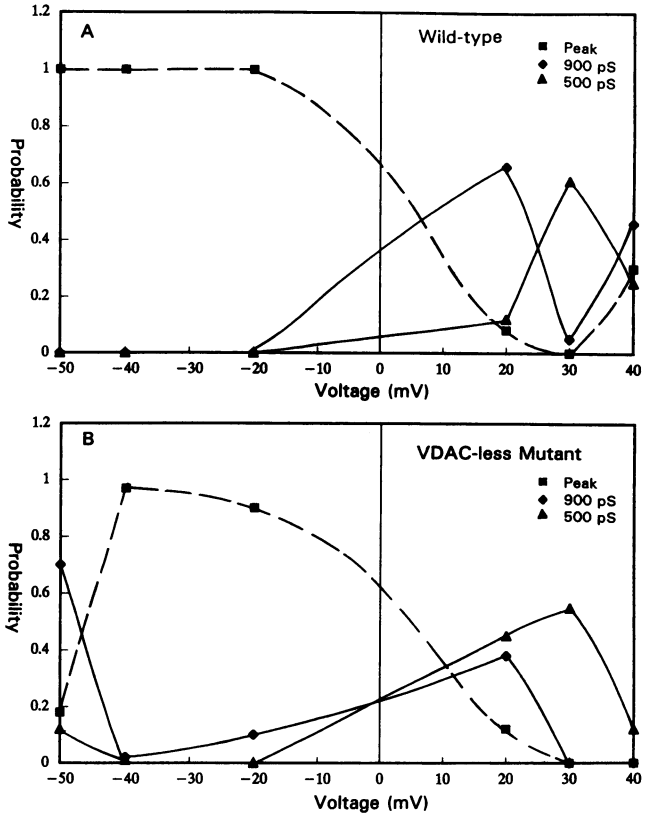

FIGURE 6 Probability of conductance level occupation is voltage-dependent. Wild-type (*A*) and VDAC-less mutant (*B*) occupancy probability for the peak (■), 900 pS (◆), and 500 pS (◆) conductance levels of MCC activity were calculated from current trace amplitude histograms as in Fig. 5.

−50 mV and amplitude histogram of Fig. 2, *A* and *B*. We found no indication that a long-lived open state exists in the mutant. Surprisingly, a long-lived closed state (C_L) was seen in the mutant at high positive potentials that was not resolved in the wild-type.

Reconstitution of MCC activity

The multiple conductance channel activity is reconstituted by fusing inner membranes with giant liposomes. Preliminary data indicate that the reconstituted channel activity (n = 8 wild-type patches (not shown) and n = 22 mutant patches, see Fig. 9) has the same peak conductance, observation of nanoSiemen transitions, selectivity, and subconductance levels as that recorded from the native mitoplasts. Furthermore, voltage profiles of the peak and subconductance levels of the mutant reconstituted activity are essentially the same as that of the native mitoplast (compare Figs. 5, 6, and 9). The slight difference in kinetics between the native and reconstituted mutant MCC activities is consistent with the variability seen in the native patches and is not considered significant.

Other yeast channel activities

A ~45 pS activity often is observed in both yeast wild-type and mutant mitoplasts that is slightly anion-selective but not

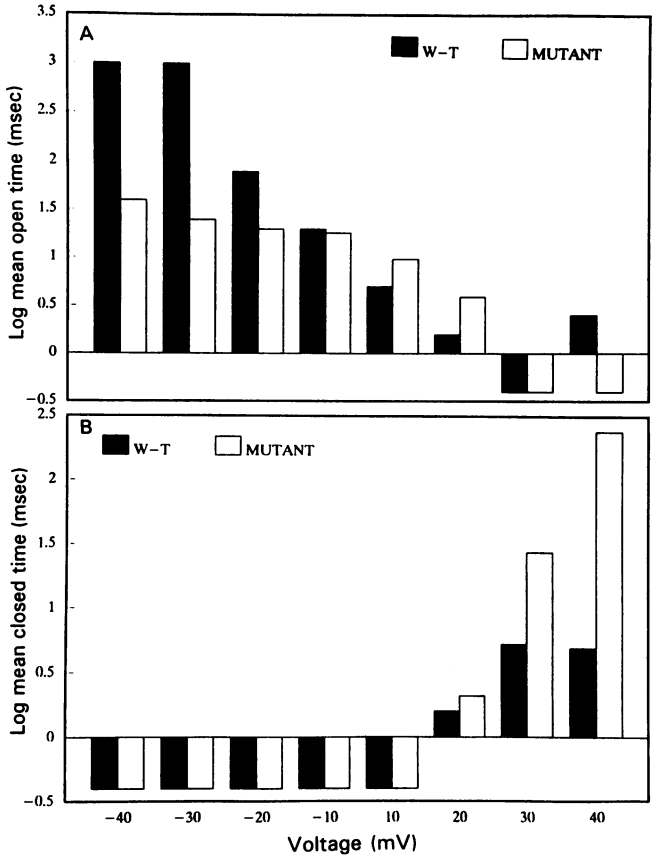

FIGURE 7 The mean open and closed times vary with applied potential. Wild-type (■) and VDAC-less mutant (□) yeast current traces were analyzed with a settling time of 0.41 ms to determine the mean times (i.e., mean duration of the fully open (*A*) and closed (*B*) states). The fully open state is not observed in either patch at 30 mV, whereas the closed state is not observed from 10 to −40 mV; these times are maximally estimated and plotted as the lowest resolvable time, i.e., −0.4 (log 0.41 ms). Other conditions as in Fig. 2.

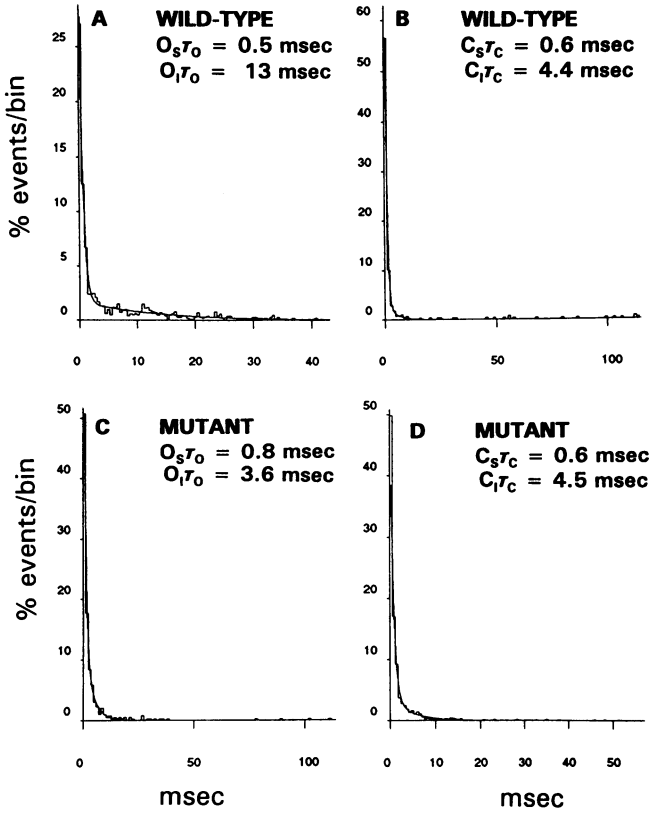

FIGURE 8 Open and closed time distributions have multiple components. (*A*) Peak conductance open time distribution from a wild-type mitoplast current trace (971 events) at 20 mV is fit by two exponentials with a settling time of 0.5 ms and reveals two decay time constants where the short duration component $O_S\tau_o$ is 0.5 ms (46% of total area) and the intermediate duration component $O_I\tau_o$ is 13 ms (52% of total area). A long duration component is visualized by the current trace of Fig. 1 at −40 and −50 mV, where the $O_L\tau_o$ is seconds. (*B*) Closed time distribution from a wild-type mitoplast current trace at 20 mV with 252 events is fit by two exponentials, where the short duration component $C_S\tau_c$ is 0.6 ms (73% of total area) and the intermediate duration component $C_I\tau_c$ is 4.4 ms (14% of total area). (*C*) Open time distribution from a mutant mitoplast current trace (390 events) at 20 mV is fit by two exponentials, where $O_S\tau_o$ is 0.8 ms (55% of total area) and $O_I\tau_o$ is 3.6 ms (45% of total area). (*D*) Closed time distribution from a mutant mitoplast current trace at 20 mV with 1332 events is fit by two exponentials where $C_S\tau_c$ is 0.6 ms (61% of total area) and $C_I\tau_c$ is 4.5 ms (28% of total area). A long duration component is visualized by the current trace of Fig. 3 at 40 mV, where the $C_L\tau_c$ is seconds.

strongly voltage-dependent (data not shown). The relationship between the activity recorded in yeast and that of the 45 pS alkaline pH-activated anion channel activity (AAA) (Antonenko et al., 1994) presently is being investigated. However, the ~100 pS voltage-dependent anion-selective activity (known as mitochondrial centum picoSiemen activity, mCS) seen in 68% of mammalian patches (Kinnally et al., 1991) has not yet been recorded from yeast mitoplasts in over 180 patches.

DISCUSSION

Mammalian and yeast mitoplasts have similar MCC activities

The properties of the mitoplast channel activities from mammalian (Kinnally et al., 1989, 1991, 1992; Campo et al., 1992; Zorov et al., 1992, and this report) and wild-type yeast are very similar. A summary of channel properties is provided in Table 1. The only statistically significant difference lies in the shift of V_0 to higher potentials in the mammalian system (Fig. 5). However, this may not be biologically significant because V_0 is a sensitive parameter. In other systems, V_0 can be quite variable (Hartshone et al., 1986) and significantly altered, e.g., simply by the presence of divalent cations (Moczydlowski, 1986). Hence, small differences in conditions might be responsible for discrepancies in V_0 between the three systems. Alternatively, the shift in V_0 may reflect some intrinsic difference in the channel protein(s). As summarized in Table 1, the correspondence in behavior includes the fact that both yeast and mammalian MCC activities have multiple subconductance levels, with transitions of

TABLE 1 Comparison of yeast and mammalian MCC activities

	Mammalian	Wild-type	VDAC-less Mutant
Multiple conductance levels	50–1500 pS	50–1500 pS	50–1500 pS
Peak conductance (pS)	1000–1500	1000–1500	1000–1500
Predominant transition size (pS)	300–500	300–500	300–500
nS transition frequency (% patches)	20 (n = 25)	19 (n = 16)	18 (n = 28)
Frequency of MCC (% patches)	76 (n = 50)	76 (n = 21)	58 (n = 48)
Selectivity: Permeability of K^+/Cl^-	3–10*	~6‡	~6‡
Gating charge§	−5.4 ± 1.0	−4.7 ± 1.1	−4.7 ± 1.3
V_o (mV)§	23 ± 3	5.3 ± 8	6.3 ± 10
Peak open probability −40 to 30 mV§	declines	declines	declines
Open probability −60 to −40 mV§	high	high	low
Multiple kinetic states	present, not quantified	5 present	5 present
Long duration kinetic state (>100 ms)	open	open	closed

*Substates up to 500 pS tested.
‡All substates including peak.
§See Fig. 5 for direct comparison.

300–500 pS predominating. Both have peak conductances of 1000–1500 pS, similar occupation of subconductance levels (Fig. 6 *A*) (Zorov et al., 1992), and a comparable voltage dependence, as reflected in open probability and gating charge (Fig. 5). Both mammalian and yeast activities have conductance levels that are only slightly cation-selective. (Note that Szabó and Zoratti (1992) found mammalian MCC activity to be unselective.) A difference noticed, but not analyzed in detail, is a higher number of subconductance levels in the mammalian mitoplasts. Although it is conceivable that these two activities represent different entities, the electrophysiological evidence strongly supports the notion that they are the same activity. Resolution of this question must await isolation of the respective proteins. It is interesting to note that although the outer membrane channel VDAC from yeast and mammals is electrophysiologically quite similar, they are biochemically distinct (DePinto et al., 1987). The conservation of MCC activity in organisms as widely separated in evolution as mammals and yeast suggests that it serves some important cellular function.

In contrast to MCC activity, we have found no evidence of the other predominant mammalian mitoplast channel activity, called mitochondrial centum picoSiemen (mCS) channel. However, this activity, like each of the other mitoplast channel activities (Kinnally et al., 1991; Antonenko et al., 1991), is recorded from mammalian mitoplasts only after activation, and the appropriate conditions for mCS activation may be different in yeast. Hence, the apparent lack of mCS may be a reflection of a lack of activating conditions. The relationship between the 45-pS anion-selective activity recorded in yeast (data not shown) at pH 7.4 and that of the AAA (Antonenko et al., 1991, 1994) is being investigated presently.

Kinetic model for yeast MCC activity

Single-channel analysis of the multiple conductance channel activity recorded from yeast mitoplasts has enabled the development of a linear kinetic model to describe MCC activity, as shown in Fig. 10. The ordering of the states is unknown. Multiple kinetic open and closed states are proposed for both the wild-type and mutant MCC activities because two to three exponentials are needed to best-fit the open and closed time distributions as has been done in other systems (Moczydlowski, 1986). This model illustrates the resolved kinetic states shared between the wild-type and mutant yeast MCC activities, as well as the states that appear to be strain-specific. These later states include a long duration open state ($O_L\tau_O$ > 100 ms) occupied at large negative potentials in the wild-type and a long duration closed state ($C_L\tau_C$ > 100 ms) occupied at large positive potentials in the mutant. The four shared states are short (τ < 3 ms) and intermediate (τ = 3–100 ms) duration open and closed states (O_S, C_S, O_I, and C_I), as shown in Fig. 8 at 20 mV. A long-lived open state (O_L) is not resolved in the mutant open time distributions, and a long-lived closed state (C_L) is not seen in the wild-type closed time distributions. Although not shown in Fig. 10, there likely is a second open state at high positive potentials in the wild-type that is energetically distinct from that seen at low positive potentials. This additional open state probably is linked in some way to the long duration open state seen at high negative potentials, because both are absent in the VDAC-deletion mutant MCC activity.

Comparison of MCC activities in wild-type and VDAC-less mutant yeast

The channel activity recorded from wild-type yeast mitoplasts has many characteristics in common with that of the VDAC-deletion mutant activity, as summarized in Table 1. These properties include: 1) a peak conductance of 1000–1500 pS; 2) the presence of multiple subconductance levels; 3) predominate transition sizes of 300–500 pS; 4) presence and frequency (18 and 19%) of nanoSiemen transitions; 5) slight cation selectivity with K^+/Cl^- permeability ratios of ~6; 6) multiple kinetic open and closed states; 7) similar voltage dependence of mean open and closed times and open probability in the range of −40 to 30 mV; and 8) existence of similar kinetic open and closed states. The open probability of the peak state is identical between the two in the general range of −40 to 30 mV. The occupancy of the same lower conductance levels (i.e., 900 and 500 pS) at low positive voltage is also very similar. The V_0 and gating charge calculated in this range are essentially the same for both mutant and wild-type activity. MCC activity is recorded from 58% (n = 48 patches) of the patches from mutant yeast

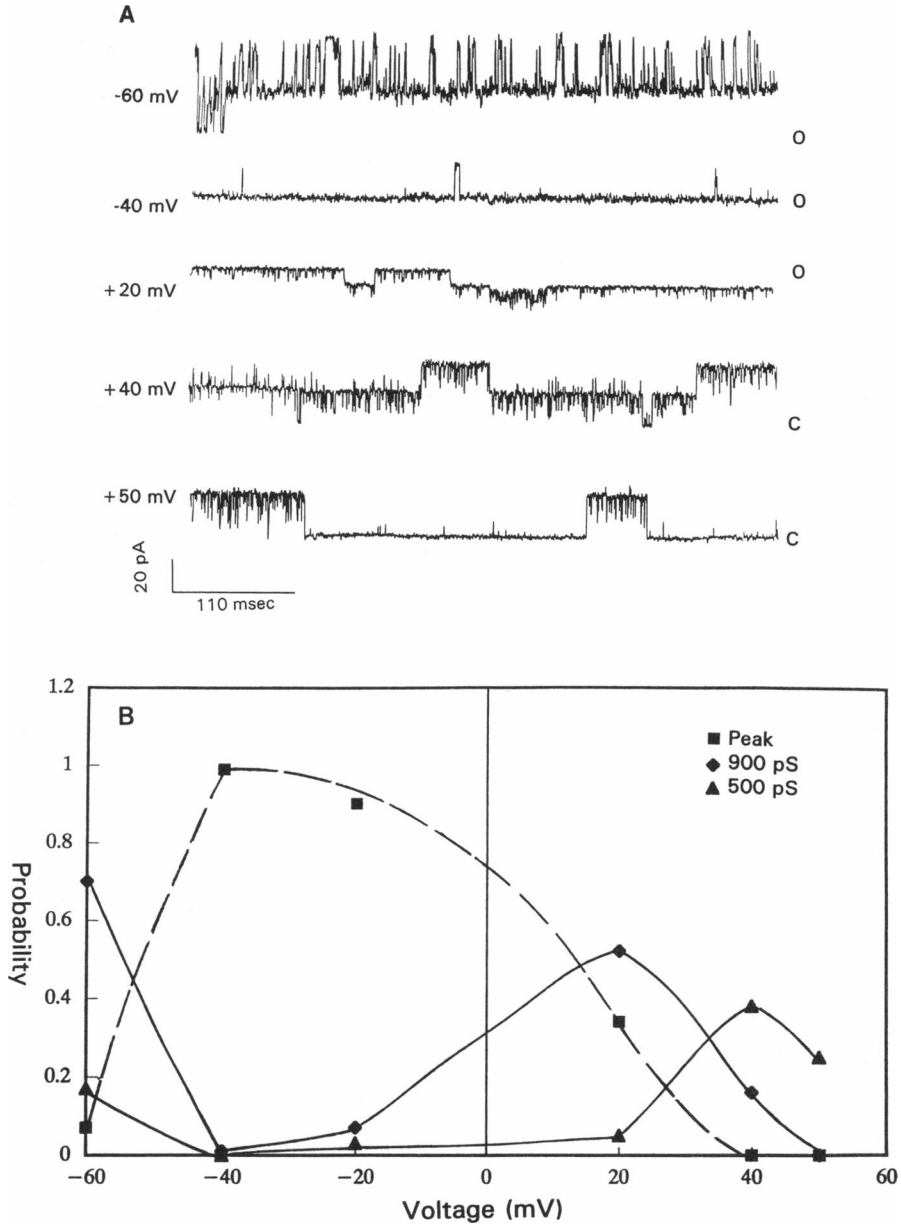

FIGURE 9 MCC activity is reconstituted from detergent-solubilized yeast mutant inner membrane fractions in liposomes. (*A*) Current traces at selected voltages from an excised patch show multiple conductance levels with dominant transitions of 500 pS. (*B*) Open probability of the fully open 1300 pS (■) and partially open conductance (900, ◆; 500, ▲) levels. Slightly higher positive voltages were needed to attain full closure, and the mean open time is slightly less in this reconstituted activity than in the native system shown in Figs. 3 and 7. However, these differences were not significant when the average behavior was considered. Other conditions as in Fig. 2.

mitoplasts, which is slightly less frequent than that seen with the wild-type (76% of 21 patches). However, the average number of channels per patch (usually 1–4) as well as the seal success rate are not dramatically different between the wild-type and mutant. These results indicate that the source of the activity likely is the same in the wild-type and VDAC-less mutant. Because our Western blot analysis shows that VDAC is not present in the mutant, our data indicate that VDAC is not required for MCC activity.

Although the MCC activities of the wild-type and VDAC-less mutant are essentially the same at low potentials, there are significant differences at transmembrane potentials outside the range −40 to 30 mV. In contrast to the wild-type MCC activity, the open probability of the VDAC-less mutant activity is reduced at larger voltages of either polarity, reflecting the occupation of either substate levels or the fully closed state. The mutant MCC activity occupies the 900- and 500-pS conductance levels at negative potentials greater than −40 mV, whereas the wild-type remains in the peak conductance level for seconds at a time. At positive potentials greater than 30 mV, mutant MCC is basically closed (occupation of conductance levels below 500 pS), whereas the wild-type mean conductance increases at high positive potentials. Fig. 10 illustrates the four kinetic states shared between the two activities, as well as the states that are wild-type- or mutant-specific. These differences, putatively attributable to the deletion of VDAC, lend some support to a role for VDAC in altering the gating properties of MCC at high transmembrane potentials. However, it is equally possible that they are related to some indirect effect of VDAC deletion. This point must await further studies.

These results agree with our previous studies implicating VDAC containing mBzR in mammalian MCC activity (Kinnally et al., 1993). They are also consistent with our proposed

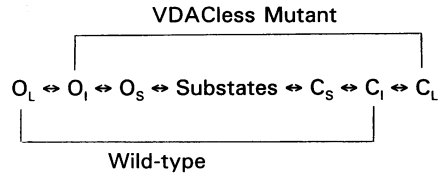

FIGURE 10 Linear kinetic model for MCC of wild-type and VDAC-less yeast mitoplasts. Analysis of single-channel fully open and closed time distributions indicate that wild-type and VDAC-less MCC exhibit at least five kinetically distinct open (O) and closed (C) states, four of which are shared and one which is strain-specific. These open and closed states are defined as: (S) short duration <3 ms, (I) Intermediate duration 3–100 ms, (L) Long duration >100 ms. Usually, 2–4 of the possible 5 states are identified at voltages between −40 and 30 mV. A $C_L\tau_c$ (long closed state) of seconds is identified in the mutant at higher positive voltages, whereas a $O_L\tau_o$ (long open state) of seconds is seen for the wild-type at higher negative potentials. See Fig. 8 for shared states.

association of MCC activity with an outer membrane component in contact sites (Kinnally et al., 1993; Kinnally and Tedeschi, 1994). Szabó et al. (1993a, b) speculated that a dimer of VDAC is responsible for MCC activity. Our results are not in apparent agreement with this speculation because MCC activity is recorded from VDAC deletion mutant mitoplasts. However, the possibility that some protein substitutes for the normal component VDAC in this mutant cannot be excluded at this time.

Reconstitution of yeast MCC activity

The ability to reconstitute VDAC-less yeast MCC activity that is not distinguishable (at the present time) from that recorded from the native membrane will be very valuable in future efforts to identify protein components. It is much easier to obtain patches in the reconstituted inner membrane preparations than the native system because, in part, of the small size of the yeast mitoplasts (0.5–1.5 μm) relative to liposomes (1–100 μm).

Physiological role of MCC activity

The tip-dip method has been used to reconstitute a peptide-sensitive channel (PSC) (Thieffry et al., 1988) from VDAC-deficient yeast (Fèvre et al., 1990, a different strain) that has channel activity similar to that of the mutant in this study. A role for PSC in protein import has been proposed because its activity is affected by signal sequences. However, the voltage dependence of the wild-type activity in our native membrane study differs from that found with the tip-dip method (Fèvre et al., 1990), possibly because VDAC activity does not survive this method of reconstitution. Nevertheless, further investigations are needed to define the relationship between PSC and MCC activities, particularly in the mammalian system. The effect of signal sequence peptides on MCC activity presently is being examined.

MCC activity's physiological role is not known, but it has been speculated to play a role in such varied processes as volume regulation, protein import, and thermogenesis. In mammals, MCC activity is thought to be responsible for the permeability transition associated with ischemia-reperfusion injury (Szabó and Zoratti, 1992; Gunter and Pfeiffer, 1990). We have proposed earlier that MCC activity is associated with contact sites between the inner and outer membranes, thereby providing some access directly between the matrix and the cytoplasm for the movement of materials (Kinnally et al., 1992; Kinnally and Tedeschi, 1994). Mammalian MCC activity is subject to regulation by a variety of physiological effectors, e.g., voltage, divalent cations, and pH. Although this electrophysiological characterization of yeast MCC activity provides a foundation, additional studies of yeast MCC activity may provide further testimony as to the identity of these activities. Yeast MCC activity may provide a less complex system than mammalian for unraveling the role of this channel activity. Ultimately, it may provide an expression system as it does already for the only mitochondrial channel that has been cloned, namely VDAC (Blachly-Dyson et al., 1990).

CONCLUSIONS

These results show that MCC activity is conserved in organisms as widely separated in evolution as mammals and yeast. The presence of VDAC is not essential for MCC activity because this activity is seen in the VDAC-deletion mutant at low transmembrane potentials. VDAC deletion does affect, through some unknown mechanism, MCC activity, and this observation is consistent with a role for VDAC in altering the gating properties of MCC activity at high transmembrane potentials.

Note added in proof—Further analysis has revealed that the differences in voltage profiles between the wild-type and VDAC-less yeast MCC are lost after reconstitution. These findings indicate reconstitution, like VDAC deletion, may disrupt factors responsible for the differences observed between the native systems.

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